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Expression of *Reticulomyxa filosa* tubulins in *Pichia pastoris*: regulation of tubulin pools

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Abstract We expressed the α2- and β2-tubulin isoforms of the giant freshwater amoeba *Reticulomyxa filosa* in the methylotrophic yeast *Pichia pastoris*. Single expression lead to little or no detectable material. Coexpression of both tubulins, however, resulted in a significant increase of expressed proteins. At the same time, the detectable internal tubulins of the host yeast cell were downregulated. This finding indicates the functionality of the expressed amoeba tubulins. Further regulation phenomena were observed on the level of equilibrium between the two *R. filosa* tubulin isoforms and on the level of the total tubulin pool. The *P. pastoris*/*R. filosa* system therefore seems to be an accessible system for the simultaneous study of the various mechanisms involved in tubulin regulation.

Key words: *Reticulomyxa filosa*; *Pichia pastoris*; Tubulin expression; Tubulin regulation; Coexpression

1. Introduction

The α-/β-tubulin heterodimer is the basic unit of the eukaryotic microtubule system which in turn is essential for cell functions such as locomotion, intracellular transport and cell division (for reviews see [1,2]). The microtubules of the freshwater amoeba *Reticulomyxa filosa* display several unusual properties including the highest known rate of assembly and disassembly [3]. These properties seem to be based, at least in part, on peculiarities of the *R. filosa* tubulins themselves [4]. Therefore we were interested in further analysis of these tubulins. Since *R. filosa* yields only moderate amounts of protein in cell culture, its tubulins have to be expressed in a heterologous system to be accessible for biochemical studies. Tubulins need to interact with eukaryotic chaperonins to gain their correct three-dimensional structure ([5–7], for a review see [8]). We therefore chose a eukaryotic system for the expression of the *R. filosa* α2- and β2-tubulin isoforms, the methylotrophic yeast *Pichia pastoris* (for reviews on *P. pastoris* see [9–11]).

Expression of heterologous tubulins has so far been reported only for mammalian [12] and insect cells [13]. We here describe the first successful expression of foreign tubulins in yeast cells as well as the first coexpression of protein subunits in the *P. pastoris* system. *P. pastoris* constitutes a tightly regulated derepression/induction system which allowed the controlled expression of the *R. filosa* tubulins. Using this system, we observed differential and coordinated regulation between heterologous and internal tubulin isoforms, between α- and β-tubulins and also of the overall amount of tubulin itself.

2. Material and methods

2.1. Vector construction

Vector pHIL-T was constructed by ligating the annealed oligonucleotides MCS1 (5′AATTAAGATACTACGCGTCCGCGGCCCGGGACTAGTCTCGAGTCCGGAT3′) and MCS2 (5′AATTATCCGGACTCGAGACTAGTCCCGGGCCGCGGACGCGTAGATCTT3′) into the *Eco*RI site of vector pHIL-D2 (Invitrogen, De Schelp, Netherlands). For the cloning of *R. filosa* tubulins, restriction sites were introduced directly adjacent 5′ to the start codon and directly adjacent 3′ to the stop codon of the respective cDNA sequences using PCR. *R. filosa* α2-tubulin (EMBL: X96476; [4]) was cloned into vector pHIL-T using *Mlu*I and *Sac*II restriction sites introduced by oligonucleotides 5′CCGTTGTTTGAACGCGTACATGCGTGAAATAAT3′ and 5′TGACCCCCCTCTCCGCGGTCATACTTCCATTTC3′. *R. filosa* β2-tubulin (EMBL: X96478; [4]) was cloned into vector pHIL-T using *Bgl*II and *Mlu*I restriction sites introduced by oligonucleotides 5′AAGAAAAGATCTATGGTGCGCAAATTG3′ and 5′CTTGGTTTTTTTTACGCGTTTAATCC3′ and into vector pPIC3K using *Bam*HI and *Not*I restriction sites introduced by oligonucleotides 5′AAGAAAGGATCCATGGTGCGCGAAATTG3′ and 5′CTTGGTTTTTTGCGGCCGCTTAATCCTCAGCTGG3′. All constructs (Fig. 1) were completely sequenced using the Sequenase 2.0 kit (Amersham, Braunschweig).

2.2. Transformation and selection of P. pastoris clones

P. pastoris strain GS115 was used for expression of the *R. filosa* tubulins. Cell culture, spheroblasting, transformation and selection of recombinant clones were performed as described in the Pichia User Manual (Invitrogen, De Schelp, Netherlands). The presence of the correct insert was verified by using direct PCR technique as described earlier [14]. Clones coexpressing α2- and β2-tubulin were generated by a first transformation with an α2/pHIL-T construct and a second transformation with a β2/pPIC3K construct. Clones derived from the second transformation were selected on YPD plates containing geneticin. Vector pPIC3K contains a neomycin phosphotransferase resistance cassette which confers resistance to 0.25 mg/ml geneticin per cassette. This relation is linear up to 12 cassettes (M. Vanetti, Invitrogen, personal communication). Clones with multiple insertions of resistance (and therefore expression) cassettes were identified by growing transformed cells on YPD plates containing multiple amounts of 0.25 mg/ml geneticin.

2.3. Antibodies and Western blots

SDS-PAGE was performed according to Garfin [15] (modified from Laemmli [16]). Western blotting was performed according to Towbin et al. [17]. Bands were visualized using peroxidase-coupled goat-anti-mouse IgG antibody (Bio-Rad, München, Germany) and the ECL kit (Amersham, Braunschweig, Germany). Antibody TAT1 (anti-*Trypanosoma brucei* α-tubulin; monoclonal, mouse; [18]) was a gift of Dr. K. Gull (Manchester, England). Antibodies WA3 (anti-bovine brain β-tubulin) and #11 (anti-*R. filosa* β2-tubulin), both mouse monoclonals, were gifts of Dr. U. Euteneuer (München, Germany). The NIH Image 1.60 program was used for measuring the signal intensities on Western blots. In the case of *R. filosa* α2 and *P. pastoris* α- and β-tubulin, amounts of expressed tubulins were estimated by comparison with respective signal intensities of defined amounts of bovine brain tubulin. Since neither *R. filosa* β2 showed crossreaction with other

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β-tubulin antibodies nor anti-*R. filosa* β2 antibody #11 crossreacted with tubulins from other sources, amounts of expressed β2 could only be estimated using the band intensities of the other tubulins as guidelines.

3. Results and discussion

3.1. *P. pastoris and R. filosa tubulins*

At the beginning of this study, biochemical or molecular biological data on the tubulins of *P. pastoris* were not available. Immediate analysis of the *P. pastoris* tubulin isoforms was therefore limited to immunological approaches. We could show that *P. pastoris* contains at least one α-tubulin that reacts with antibody TAT1 on Western blots (Figs. 2A and 3A) and one β-tubulin that is recognized by antibody WA3 (Fig. 3C). Whether these isoforms represent all α- and β-tubulins of this yeast remains to be shown. However, *S. cerevisiae*, as a comparable organism, contains only one β- [19] and two α-tubulins [20]. Additionally, antibody TAT1 is known to react with almost every α-tubulin it was tested against (Keith Gull, Manchester, England, personal communication). These facts make it likely that most, if not all, of the *P. pastoris* α- and β-tubulins were detected in our experiments.

R. filosa β2 could be easily distinguished from the yeast β-tubulin because it reacts with the #11 antibody which does not recognize *P. pastoris* β-tubulin. The yeast β-tubulin, in turn, can be detected by antibody WA3 which shows no cross-reaction with β2. On the other hand, *R. filosa* α2- and *P. pastoris* α-tubulin are both recognized by the TAT1 antibody. However, they can still be distinguished by their different migration behaviour in SDS-PAGE (Fig. 2A).

3.2. *'Single' and cotransformation*

In a first step, *P. pastoris* cells were transformed with α2 or β2 'single' constructs. Induced cultures of these transformants showed little (in the case of α2; Fig. 2A, lane 5) or no detectable expression (in the case of β2; Fig. 2B, lane 6) of heterologous tubulins. In a second step, a clone already carrying the α2-sequence was transformed with a β2-construct. The resulting cotransformants showed significant expression both of α2 and β2 (Fig. 3): 1 g of cotransformed *P. pastoris* cells expressed approximately 180 μg of α2 at an induction time of 3 days. Peak amounts were in the range of 400 μg/g cell mass. The amount of expressed β2-tubulin was estimated to be in the same range as the one of α2 (for comparison: 1 g of *S. cerevisiae* cells contains 200–700 μg of tubulin [21,22]).

Since the cotransformants were descendants of the 'single' α2-clone, the increase in α2-expression could be directly linked to the presence of the β2-construct. We therefore conclude that the presence of the β2-construct was both necessary

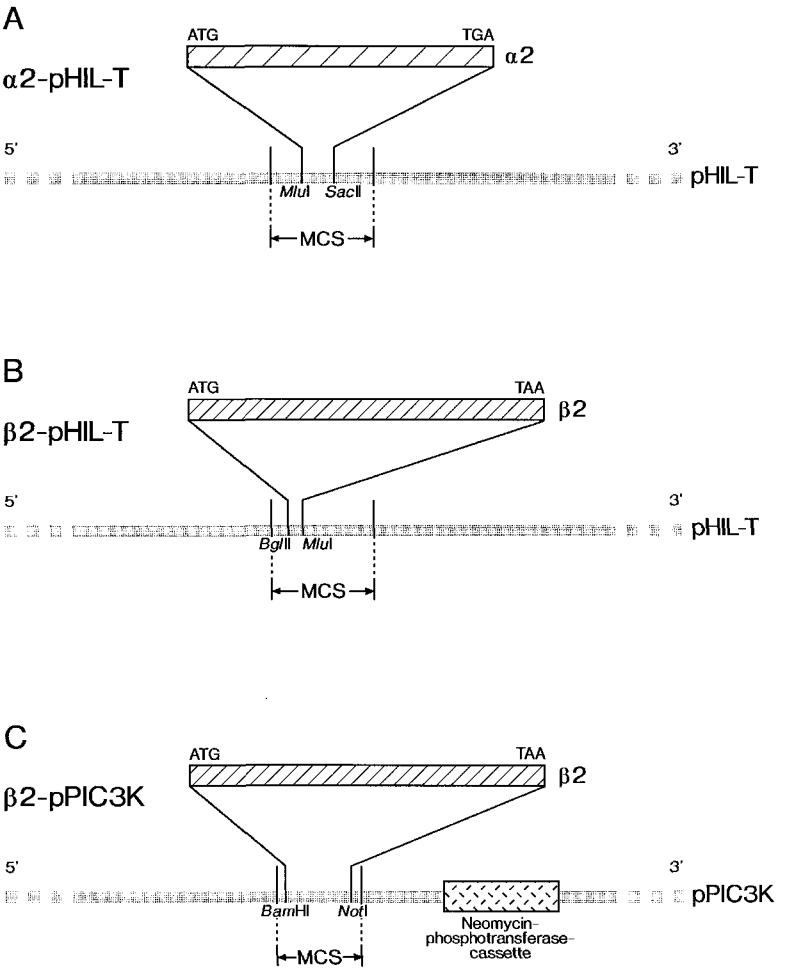

Fig. 1. Vector constructs used for expression of *R. filosa* tubulins.

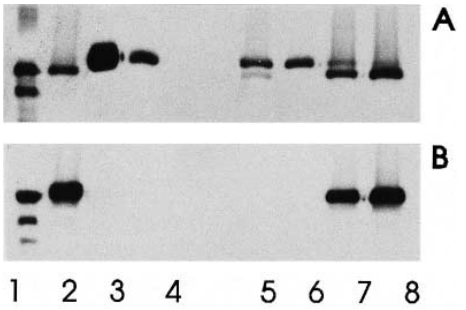

Fig. 2. Migration behaviour of *R. filosa* tubulins in SDS-PAGE and single expression versus coexpression of *R. filosa* tubulins. Western blot, developed with anti-α-tubulin antibody TAT1 (A) or anti-*R. filosa* β2 antibody #11 (B). Lane 1: *E. coli* clone coexpressing α2- and β2-tubulin; lane 2: *P. pastoris* clone coexpressing α2- and β2-tubulin and containing 3-β2-expression cassettes; lane 3: pig brain tubulin; lane 4: *P. pastoris* clone carrying only expression vector pHIL-D2; lane 5: *P. pastoris* clone transformed with α2/pHIL construct; lane 6: *P. pastoris* clone transformed with β2/pHIL construct; lane 7: *P. pastoris* clone cotransformed with α2/pHIL and β2/pPIC constructs, carrying 1 β2-expression cassette; lane 8: *P. pastoris* clone cotransformed with α2/pHIL and β2/pPIC constructs, carrying 3 β2-expression cassettes.

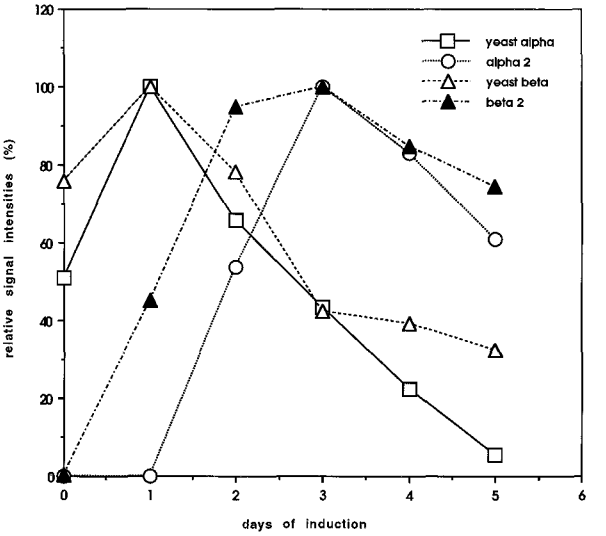

Fig. 4. Time course and amounts of expressed tubulins. Relative signal intensities from Western blot in Fig. 2 are plotted against days of induction. For each tubulin isoform, maximum of expression was set as 100%.

and sufficient for the raised expression levels of α2. A similar conclusion can be drawn in the case of the heightened β2-expression. However, since the β2-constructs used for 'single' or 'double' transformation are not identical (the β2 'single' construct is based on another vector and has 6 additional bps 5′ of the start ATG), this conclusion is not as stringent. Alternatively, the raised β2-expression levels could be due to an integration of a higher number of β2-expression cassettes in the cotransformed clones. However, this could be excluded since cotransformed clones with only one β2-cassette – as determined by their resistance to geneticin (see Section 2) – showed a similar rise in expression.

In α2/β2-cotransformed clones themselves, expression levels were further influenced by the number of β2-expression cassettes present. A cotransformed clone carrying 3 cassettes of β2 ('3-β2') expressed more β2-protein than a clone containing only 1 cassette ('1-β2'; Fig. 2B, lanes 7 and 8). The observed relationship between the number of expression cassettes and the amounts of expressed protein was not linear (expression cassettes: factor 3; expressed β2-protein: factor 1.5–2). At the same time, the 3-β2-clone also expressed more heterologous α2- and less internal α-tubulin (Fig. 2A, lanes 7 and 8). These findings are in contrast to earlier studies in *S. cerevisiae* [23,24] where clones carrying cassettes theoretically sufficient for the production of a 2-fold excess of tubulin isoforms showed only a 15% elevation in tubulin expression. This difference in expression regulation between the two yeast species may be due to differences in the constructs used for overexpression but it may also be due to a different stringency of the respective regulation mechanisms involved. In the case of β2, the latter may at least in part be based on aberrant sequence motifs important for regulation [4]. However, since also the highly homologous α2 shows similar behaviour in these experiments, sequence aberrancy in itself is probably not the decisive factor for the observed difference.

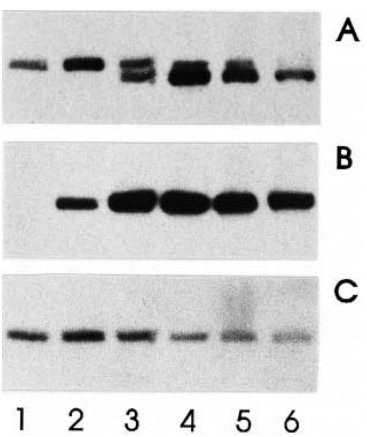

Fig. 3. Time course of tubulin expression. Western blot of *P. pastoris* clone coexpressing *R. filosa* α2- and β2-tubulins and containing 3 β2-expression cassettes, developed with anti-α-tubulin antibody TAT1 (A), with anti-*R. filosa* β2-tubulin antibody #11 (B) or with anti-β-tubulin antibody WA3 (C).

3.3. Dynamics of tubulin expression

Regulation of tubulin levels in the cell has been shown to be a complex phenomenon ranging from differential gene activation in lower eukaryotes [25–27] to autoregulation in animal cells [28]. An important point seems to be the maintenance of stoichiometric amounts of α- and β-tubulin [29–31]. On the other hand, there is evidence that the regulation of α-tubulin is independent from that of β-tubulin [32]. To follow the dynamics of tubulin expression in the *P. pastoris*/*R. filosa* system, an induction time course of a cotransformed 3-β2-clone was made (Fig. 3). Several regulation phenomena could be observed: increased expression of the *R. filosa* tubulins was coupled with decreased expression of the internal yeast tubulins until only the *R. filosa* tubulins could be detected (Fig. 3A and C, lane 6). This was probably due to a relatively fast acting transcriptional or translational regulation mechanism responsible for keeping the overall amount of α- and β-tubulin, respectively, constant (*regulation I*). It may be analogous to the translational repression of tubulin observed in CHO cells [33]. This finding also suggests that the expressed *R. filosa*

tubulins were functional and able to substitute for the internal *P. pastoris* tubulins. However, the possible involvement of additional isoforms of *P. pastoris* tubulins that were not detected by the antibodies used, cannot be ruled out.

Another kind of regulation seemed to be keeping expression levels of the α2- and β2-tubulins in the same range with respect to each other (*regulation II*): increase, peak and decrease of expression of both isoforms were nearly parallel (Fig. 4). However, α2 was detectable only with β2-tubulin already present. This points to an influence of β2-tubulin on the expression of α2. A possible explanation is the interaction of β2 with an otherwise unstable α2-molecule. A similar model was proposed for *S. cerevisiae* [24,33]: single α-tubulin should be quickly degraded and does not accumulate. It is also expressed in slight excess over β-tubulin which acts as a cytotoxin when it is undimerized. The low expression of α2 in the α2 'single' clone (i.e. without overexpression of β2 in parallel) may be another manifestation of regulation mechanism I. A combination of mechanisms I and II becomes apparent upon comparing the 1-β2- and 3-β2-clones (Fig. 2): an increase of expressed β2 is accompanied by an increase of α2-protein. At the same time, expression of the internal yeast α-tubulin is lowered.

A third form of regulation concerned the total pool of internal and heterologous (α- and β-) tubulin over a period of several days. After initial overexpression, the amounts of *R. filosa* tubulins (which by then represented the biggest fraction of detectable tubulins) were subsequently lowered until they reached about the same levels as that of *P. pastoris* tubulins in uninduced cells (*regulation III*; Fig. 3A and B).

These observations clearly show that the regulation of tubulin expression in *P. pastoris* is composed of several mechanisms that sometimes act simultaneously. Similar observations were made in other cell types [23,24,29,32], but – due to the biochemical and immunological differences of the tubulins studied – this is the first time that the concerted action of individual regulation mechanisms could be shown.

Additionally, in comparison to other tubulin expression systems, the *P. pastoris*/*R. filosa* system exhibits several unique properties: (1) *R. filosa* tubulins accumulate to a significant extent only in coexpression. This is in contrast to earlier studies in *S. cerevisiae* [23] and in CHO cells [29]. In these studies, overexpression of one (non-heterologous) subunit also induced overexpression of the other (non-heterologous) subunit. The difference to the *P. pastoris*/*R. filosa* system is probably due to the absence of non-coding sequences of possible regulatory relevance in the *R. filosa* constructs. Expression regulation of the (heterologous) *R. filosa* tubulins can therefore occur only at the protein level, and regulation mechanisms that act before this level cannot take effect. Transcriptional/translational induction and protein stabilization effects can therefore be clearly separated in this system. (2) The maximum of overexpression that was achieved for both *R. filosa* tubulins was only about 5-fold compared to the normal level of *P. pastoris* cells, whereas overexpressed *S. cerevisiae* tubulins accumulated up to 64-fold [23]. (3) Effects such as growth impairment, cell cycle arrest or lethality that occur upon a 1.4-fold overexpression of a single β-tubulin [24] or a 32-fold overexpression of both α- and β-tubulin [23] in *S. cerevisiae* were not observed here. Conceivably, because the *R. filosa* tubulins accumulate to a significant extent only in coexpression where toxic effects of a single β-tubulin are suppressed by dimerization and also because the heterologous tubulins do not exceed amounts that are five times higher than the normal levels of *P. pastoris* tubulins (Fig. 3, lanes 1 and 4). Possible destabilizing effects on microtubules because of the relative lack of microtubule associated proteins [34] were therefore probably not as severe as in cells containing grossly overexpressed amounts of tubulins. (4) At the same time, *P. pastoris* has a more productive ratio of gene copy number to tubulin expressed (3: 1.5–2) compared to *S. cerevisiae* or CHO cells (2: 1.15 [24]). *P. pastoris* therefore seems a stable and efficient system for overproducing tubulins and also for the study of the diverse and complex mechanisms involved in tubulin regulation.

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